Curr Microbiol (2008) 56:224–228
DOI 10.1007/s00284-007-9061-0

A Gene Having Sequence Homology to Isoamyl Alcohol Oxidase Is Transcribed During Patulin Production in *Penicillium griseofulvum*

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Received: 13 September 2007 / Accepted: 24 September 2007 / Published online: 14 November 2007

Abstract The genes for the patulin biosynthetic pathway are most likely arranged in a cluster, as is often the case for other mycotoxins. With this in mind, GeneWalking has been performed to identify genes both upstream and downstream of the isoepoxydon dehydrogenase (*idh*) gene. A gene present in *Penicillium griseofulvum* NRRL 2159A had high sequence homology to the isoamyl alcohol oxidase (*iao*) gene and was detected downstream of the *idh* gene and in the same orientation. By virtue of the presence of a signal peptide sequence, the newly identified gene coded for a secreted protein with an FAD-binding domain and potential for *N*-glycosylation. An open reading frame consisted of 1946 nucleotides, containing four putative introns and encoding a 22 amino acid signal peptide. The 571 amino acid mature protein contained nine cysteine residues and had 11 potential *N*-linked glycosylation sites. Searches using GenBank indicated that *Aspergillus terreus*, *A. oryzae*, *A. fumigatus*, and *Gibberella zeae* contain genes coding for a putative isoamyl alcohol oxidase. When the translated query was compared with the translated database, the highest scores were seen with *A. clavatus* (E value of 0.00), *A. fumigatus* (E value of $8e^{-142}$), and *A. oryzae* and *A. terreus* (each having an E value of $2e^{-141}$). Reverse transcription-polymerase chain reaction analysis confirmed that the *iao* gene was transcribed. The amplified products were sequenced for confirmation of their identities. This is the first report of an isoamyl alcohol oxidase gene in a species of the genus *Penicillium*.

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Introduction

Fungal genes governing production of aflatoxins, fumonisins, and zearalenone are frequently found in clusters on chromosomes. Gene clusters have been described for *Aspergillus flavus*, *Fusarium verticilliodes*, and *Gibberella zeae*, which are responsible for biosynthesis of the mycotoxins aflatoxin, fumonisin, and zearalenone, respectively [9, 10, 11, 12, 19]. Biochemical studies of enzymes involved in patulin biosynthesis have been reported [2, 13, 14]. The research presented here was undertaken to determine whether there were genes near the isoepoxydon dehydrogenase (*idh*) gene coding for other enzymes necessary for the synthesis of patulin. Until recently, only two genes in the patulin biosynthetic pathway had been described. These genes were the first enzyme in the pathway, 6-methylsalicylic acid synthase (*6-msas*) [2] and the seventh in the pathway, isoepoxydon dehydrogenase (*idh*) [8]. Information on the genes in the patulin biosynthetic pathway has been expanded in a recent publication, in which additional genes clustered upstream of the *idh* gene of *Penicillium expansum* were described, including a cloned 715-bp fragment of a putative ATP-binding cassette transporter gene *peab1* and partial sequences of two putative cytochrome P450 monooxygenase genes *P-450 1* and *P-450 2* [15].

Mycotoxins can contaminate agricultural products both before and after harvest. The mycotoxin patulin is produced by a number of *Penicillium* species. *Penicillium expansum* is the species commonly associated with patulin production in apple products. The focus of the research reported here was that *Penicillium griseofulvum* NRRL 2159A, capable of producing patulin, has a putative *iao* gene that codes for isoamyl alcohol oxidase. Fungi containing putative *iao* genes include *Aspergillus terreus*

(GenBank accession no. XM_001209796), *A. oryzae* [17, 18], *A. fumigatus* (GenBank accession no. XM_742622), and *Gibberella zeae* [9, 10].

Materials and Methods

Fungal Strain, Culture Conditions, and DNA Extraction

Culture conditions and isolation of genomic DNA of *P. griseofulvum* NRRL 2159A were performed as previously described [7]. Polymerase chain reaction (PCR) amplification and cleanup of amplified DNA were performed as described previously; nucleotide sequencing of PCR products for both strands was performed using an Applied Biosystems MicroAmp 96-well plate and the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) [6]. To isolate clean genomic DNA for GeneWalking, the DNA was extracted using the DNeasy Plant Mini kit from Qiagen (Valencia, CA) [7].

GeneWalking in Genome Walker Libraries

GenomeWalker™ Universal Kit (Clontech Laboratories, Mountain View, CA) was used to determine the complete sequence of the putative isoamyl alcohol oxidase downstream (3′) of the *idh* gene and in the same orientation and was performed according to manufacturer's specifications. The user manual for the GenomeWalker Universal Kit is available online (http://www.clontech.com). For the construction of the library, five separate reactions were set up for digestion of genomic DNA. Digestion of genomic DNA, purification of DNA and ligation of genomic DNA to GenomeWalker adaptors were performed according the manufacturer's specifications [7].

Primer Design for GeneWalking and Symmetrical Amplification

Two gene-specific primers were designed for each walk, one for primary PCR (GSP1) and one for secondary PCR (GSP2), based on the sequence of *P. griseofulvum* NRRL 2159A. A total of 4 gene walks were completed to obtain the entire *iao* sequence (Table 1). As indicated in Table 1, primers were designed based on the sequence of the 5′ end of the *idh* gene of *P. griseofulvum* NRRL 2159A (GenBank accession no. AF006680) and the *iao* gene of *P. griseofulvum* NRRL 2159A (GenBank accession no. EF202832). To verify the sequence of the *P. griseofulvum* NRRL 2159A *iao* gene, primers (Table 2) were designed for symmetric amplification of the *iao* gene for sequencing both strands of the *iao* gene [6]. Locations of the sequences used for designing the primers are shown in Table 2.

Agarose Gel Purification

Multiple bands of varying molecular weight were observed from the PCR products. Gel purification of each band was done using an UltraClean™ 15 DNA Purification Kit from MoBio Laboratories (Carlsbad, CA) to see whether they were part of the sequence. Carefully and quickly over a UV light box, each band was cut out of the gel and processed with the kit reagents (UltraSALT, UltraBIND and UltraWASH). The DNA was then eluted from the silica using H_2O, and purified products were cycle sequenced as described previously.

Reverse Transcription-Polymerase Chain Reaction Analysis

Cultures were grown in potato dextrose broth containing manganese chloride (152 μM) as described [5]. Total RNA

Table 1 Polymerase chain reaction primers used for Gene Walking downstream of *idh* gene

Name	Sequence (5′–3′)	Positions
Pg1-GSP1-d	GTCCCTGGGAACTCTATTCAAAGATC	2733–2758[a]
Pg1-GSP2-d	GACTGAAGGTTCCTCTGAAGCGGTTG	2784–2809[a]
Pg2-GSP1-d	GAACGGGAGGCTACCATCGGAGTTCTG	3501–3527[a]
Pg2-GSP2-d	CAGCTATCGGAGACAGAATATGCTTC	3646–3671[a]
Pg3-GSP1-d	CAATCTGTCCTAGTTATGCGGGAAAGTC	566–593[b]
Pg3-GSP2-d	CTGAACTATACCAGCCCGCACTACAC	649–674[b]
Pg4-GSP1-d	CAGCAGTTGACGCGAAGAAATTGCTG	1181–1206[b]
Pg4-GSP2-d	GTCGGCCTCGTATTTCCAGCATTACATG	1266–1293[b]

[a] Positions refer to GenBank accession no. AF006680

[b] Positions refer to GenBank accession no. EF202832 (shown in Fig. 1)

Table 2 Primer design for symmetric amplification of the complete *iao* gene

Name	Sequence (5′–3′)
IAO3844F	GATCTCAGAGTTGCTAGAC
IAO4579R	CTCGTTGATGTCTGTACCTTG
IAO4451F	GTTATGCGGGAAAGTCAACAGG
IAO5320R	ACAGATGACGAGACATTGAG
IAO5203F	AACGCTCAATACGGTGGTC
IAO6018R	AGTATTTGGCAGGGCTACTC

was extracted using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA), based on a protocol described for purification of total RNA from plants and filamentous fungi. To remove any DNA contamination from the RNA sample, the Turbo DNA-free Protocol (Ambion, Austin, TX) was used. To convert RNA into cDNA and amplify the cDNA into DNA, the Easy-A One-Tube RT-PCR System (Stratagene, La Jolla, CA) was used. Primers IaoRT3F-ACTGAC ACTGCCTGCTCTTAC and IaoRT3R-ATCCTCAACTT GCCAGTCTTC were designed to have two introns between the primer pair for the *iao* gene. Additionally, primers Idh2050F-CAACTGGTCTCAAAGGTGCC and Idh2667R-TGGGACAATTCCTGAACATGC were used

Fig. 1 Nucleotide sequence of the isoamyl alcohol oxidase (*iao*) gene of *Penicillium griseofulvum* NRRL 2159A (GenBank accession number EF202832)

Complete *P. griseofulvum* isoamyl alcohol oxidase gene

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   1 atgcgtcttc attggtcccc acgtcttctc ttgttaggca tcttgtctgt ttcgcaggtg
  61 tctgccggcc tgtcctcgag ctgccgatgt atgcccggtg atagttgttg gccgtcctta
 121 gatgactggg cacgcttcaa tacatcgatt ggcggacgtc tagtggacac gcaaccactg
 181 ggacagccct gtcatgaccc attctattct gccagtgagt gtcgtgctct gagagagcag
 241 tggaccaatc cagagatcca gtaagtaatc caacctatcc aaccagcctc atcactggcc
 301 tgctaaatca atcccctcca atagcgatgc atcatcctct tccatcatgg cagccgccgt
 361 ggccaacgaa acttgcgatg cttttggtcc ccaatcaaag ccctgcaagc tgggtgccat
 421 ggtgcgctat gccgtcaacg ccagctcccc agacgacttc atccaaacca ttcgattctc
 481 ccaggaacga gacattcgtc tcgtcattcg caacacaggc catgagtata tatcttccgg
 541 tacgaaagac atatcccctc gctaacaatc tgtcctagtt atgcgggaaa gtcaacaggt
 601 gcaggcgcac tatccatctg gacccattat gtcaaggaca ttaacttcct gaactatacc
 661 agcccgcact acacaggacc agcggtccgg atgaccgcgg gtatccaagg tacagacatc
 721 aacgaggcgg cccataaaaa aggcctggta atcgtaggcg gggaatgtgc cactgtcggc
 781 cccgtcgggg gcttcacgca aggaggaggc cactcagctc tgagctctcg gttcggtctt
 841 ggagccgacc aggtgctgga atgggaggtg gtagatggaa tgggacgatt actaactgca
 901 tcacccaccg agaacccaga tttgtactgg gctctcagcg gaggtggagg cgggacatat
 961 ggcgtcgtgt acgccatgac cgtcaaagcg tttcccgact tccccgtcac aggcgtcgtt
1021 cttgaattca agaacaaaaa tccctcgtcg gaccgtttct tccaagccgt aggccattac
1081 catcgtcacc tgcctacgta tactgcggct ggtgggatgg gaattgcaca aatcacaaac
1141 tcctcgttcc tgctcacccc actgacactg cctgctctta cagcagttga cgcgaagaaa
1201 ttgctggcac catttttgga tgatctgcgt gggctgaaca tttcttatac actgaacatc
1261 acccagtcgg cctcgtattt ccagcattac atgaaactga tcgagcccaa cccgactcaa
1321 ctggtgcaaa acgctcaata cggtggtcgt cttcttccgc tcgatgtcat tcagaataat
1381 aacacccagc tcacggatgc ggtccggaaa atcaccgagg atggggctat cttcgtgggc
1441 attggtctca atgtctcgtc atctgtgacc ggcaatttgt ggaattcagt tcatcctgca
1501 tggcgtacag cagcaatgac tgtcatatta tctacgtacg ctctcatccc tgacgtgatg
1561 gttgcctttg tttcaatata ctcactgact ctacaggaac tggcccgctg gtgcgaacct
1621 cgctgagatg aaaacgctgg caaataaaat gacgacaaaa tgggtaccgc tcctaaccga
1681 cctgagtcca gattcgggtt gttatatgag cgaggcaagg agaaatacat tcctgatttg
1741 gaaatcacta gctaactatc acctaggctg atccccagca accaaattgg caacacacct
1801 tttacggtcg caactataat tcgttgtacg cgatcaagaa gaaatatgat ccgttccaga
1861 cgttctatgc tacgacagca gtgggcagtg aagactggca agttgaggat ggtggtcgtt
1921 tgtgccaggc caccagaatg aattga
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P.griseofulvum iao      MRLH-WSPRL LLLGILSVSQ VSAGLSSSCR CMPGDSCWPS LDDWARFNTS IGGRLVDTQP
A.clavatus FAD binding  ...SIY.SI. ..RAMCL.RP TFGFPATT.. .......... ST...H.NA. .....IA...
A.oryzae mreB           .FGQ-VTGLS ..T.L.ATAS .K.--DSN.. .F...A...A Q.V..K.NE. VD....A.V.   60

P.griseofulvum iao      LGQPCHDPFY SASECRALRE QWTNPEIHDA SSSSIMAAAV ANETCDAFGP QSKPCKLGAM
A.clavatus FAD binding  .A.A....Y. NET..QY.QK H..L.AL..I .P........ .KD.....T. R....AP.D.
A.oryzae mreB           ..T.....N. N.A..QK.S. ...D.AL.YE T......PWF TNG...P.H. E....TI.NY  120

P.griseofulvum iao      VRYAVNASSP DDFIQTIRFS QERDIRLVIR NTGHDYAGKS TGAGALSIWT HYVKDINFLN
A.clavatus FAD binding  .V.S.N.... ...SR..... .Q.N...... ......L... .......... ..L...E.VN
A.oryzae mreB           .V...DVAK. EHVSTALK.A K.HN..V.P. ......N... ......A..M .HI...EIKD  180

P.griseofulvum iao      YTSPHYTGPA VRMTAGIQGT DINEAAHKKG LVIVGGECAT VGPVGGFTQG GGHSALSSRF
A.clavatus FAD binding  ...SS..... FT.A..V..S ..YNV.NGR. ..V......S ......Y... ..........
A.oryzae mreB           .KDT..Q.K. IK.G..V..G EAY..GYNA. .QV.....P. ..IA..Y... .........Y  240

P.griseofulvum iao      GLGADQVLEW EVVDGMGRLL TAS-PTENPD LYWALSGGGG GTYGVVYAMT VKAFPDFPVT
A.clavatus FAD binding  ..A....... Q....T.... ...-..Q... .......... .......S.. ..........
A.oryzae mreB           ......A... ..I..E.NFI ..TRDN.YS. .......... .S..ITWSL. A..HTGT..S  300

P.griseofulvum iao      GVVLEFKNKN PSSDRFFQAV GHYHRHLPTY TAAGGMGIAQ ITNSSFLLTP LTLPALTAVD
A.clavatus FAD binding  ....Q.DT.N T..KD..... SY...D.... ......A... ..R..... ....NK.TEE
A.oryzae mreB           .YN.S.T.DG M.Q.T.YE.. SLWQTV..SV VD..A.AVWM F.NT..MI.. ..G.NIPVA.  360

P.griseofulvum iao      AKKLLAPFLD DLRGLNISYT LNITQSASYF QHYMKLIEPN PTQLVQNAQY GGRLLPLDVI
A.clavatus FAD binding  .RS.I...IH E.ET.H.P.Q .N.....T.L E..K.....N .......G.. .......N..
A.oryzae mreB           LEA.VK..T. G.TK.G.T.. TYSK.FD..L EEFNAM---Q GAIE.AT... ..W.I.RS.V  420

P.griseofulvum iao      QNNNTQLTDA VRKITEDGAI FVGIGLNVSS SVTGNLWNSV HPAWRTAAMT VILSTNWPAG
A.clavatus FAD binding  ESN.....E. .KT..Q..VV ......N... ..V.DV.... L...... .L S.L...D...
A.oryzae mreB           E.N.DG..A. Y.H......T .I.V..N..K ALV.DVD.A. L....ETLIH STIT.P.KWN  480

P.griseofulvum iao      ANLAEMKTLA NKMTTKWVPL LTDLSPDSGC YMSEADPQQP NWQHTFYGRN YNSLYAIKKK
A.clavatus FAD binding  ..RST..... DR..S....I ..A....... ..N..... D.PQ..... .ET.....R
A.oryzae mreB           .R-S..LAEQ D...NDYISA ..KVA.N..A .LN...FR.. .F.KY...D. .AT.RK..A.  540

P.griseofulvum iao      YDPFQTFYAT TAVGSEDWQV EDGGRLCQAT RMN  571
A.clavatus FAD binding  ....D....S .....G.... KTD....RVK GNT  572
A.oryzae mreB           ...DNL.... .....DE.T. RED....SV       563
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Fig. 2 Alignment of deduced amino acid sequences of the isoamyl alcohol oxidase (*iao*) gene for *Penicillium griseofulvum* NRRL 2159A (GenBank accession no. EF202832), *Aspergillus clavatus* (GenBank accession no. XM_001273096), and *A. oryzae mre*B (GenBank accession no. AB085788). Asparagine residues in the three proteins that are potential sites of N-glycosylation are shown as **N**. There are 571 and 563 amino acids present in the IAO proteins of *P. griseofulvum* NRRL 2159A and *A. oryzae*, respectively, and 572 amino acids present in the FAD binding protein of *A. clavatus*

to amplify the *idh* gene. The amplified products were purified and sequenced.

Results and Discussion

The nucleotide sequence of the *iao* gene (Fig. 1) was determined for *Penicillium griseofulvum* NRRL 2159A. In order to determine the nucleotide sequence of the *iao* gene, sequencing was first done by GeneWalking and then new primers were designed to determine by symmetric amplification the sequence of the *iao* gene in both directions. The 5′ end of the nucleotide sequence of the *iao* gene was located in the 3′ UTR of the *idh* gene [8], starting at nucleotide 3874 (ATG) of the *idh* sequence and occurring in the same orientation; the TATAA sequence was located at *idh* nucleotides 3798–3802. The *iao* open reading frame, consisting of 1946 bp, was interrupted by four putative introns, and it encoded a 22 amino acid signal peptide [3] and a 571 amino acid mature protein. The newly identified *iao* gene coded for a secreted protein with an FAD binding domain (FAD_binding_4 family: pfam01565.11). Most of the enzymes in this family are similar to oxygen oxidoreductase. The mature protein contained nine cysteine residues and 11 potential *N*-linked glycosylation sites, determined as described [20]. The IAO protein is likely to be secreted because it does not contain an "endoplasmic reticulum retention signal," which would be expected at the C terminus of the mature protein [3]. In putative introns 1–3, canonical splice sites (GT-AG) were observed. Putative intron 4 had a splice site (GC-AG), which is characteristic of a small number of eukaryotic introns [4] and was present in the fatty acid synthetase (*FAS2*) gene from *Penicillium patulum* strain DSM62862 [16]. Multiple noncanonical polyadenylation sites were present in the

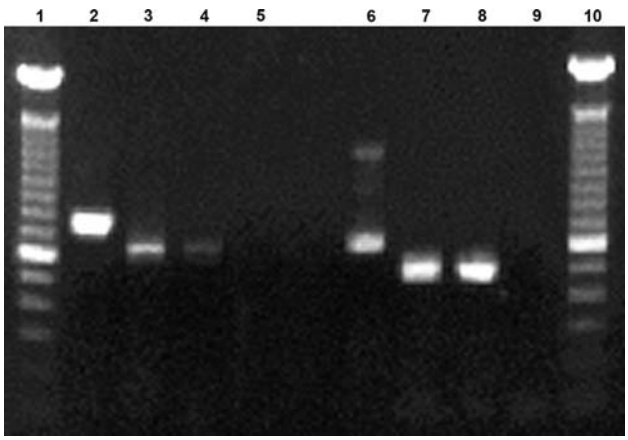

Fig. 3 Reverse transcription-polymerase chain reaction of *iao* and *idh* gene transcripts in different culture conditions, compared with their respective DNA transcripts. *Penicillium griseofulvum* NRRL 2159A was cultured for 48 h or 96 h. Lanes: (1, 10) molecular weight markers; (2) genomic DNA from gene *iao* template, resulting in a 750-bp product; (3, 4) cDNA amplicon from *iao* gene is 637 bp long, at 48 h and 96 h, respectively; (5, 9) control with no RT added; (6) genomic DNA from *idh* template, resulting in a 618-bp product; (7, 8) cDNA amplicon from *idh* gene is 511 bp long, at 48 h and 96 h, respectively

3′-noncoding region of the putative *iao* gene; mRNAs with multiple poly (A) sites tend to use noncanonical polyadenylation signals more often than mRNAs with a single poly (A) site [1].

The deduced amino acid sequence of the *iao* gene determined here was compared with the protein sequences of isoamyl alcohol oxidase present in GenBank. Isoamyl alcohol oxidase is a novel enzyme that catalyzes the formation of isovaleraldehyde, a main component of *mureka* that gives sake an off-flavor [18]. The translated sequences of the *P. griseofulvum iao* gene and the putative isoamyl alcohol oxidase gene of *A. oryzae mre*B were aligned; several (5) of the potential N-linked glycosylation sites are aligned (Fig. 2). Also included in the alignment is the recently reported sequence of *Aspergillus clavatus* NRRL 1 FAD binding domain protein (GenBank accession no. XM_001273096), which has high similarity to putative isoamyl alcohol oxidase genes and is located 3′ of the *idh* gene in that particular organism, as it was in *P. griseofulvum*. Another gene, zearalenone biosynthesis gene 1 (ZEB1), shows a high similarity to putative isoamyl alcohol oxidase genes and is responsible for the chemical conversion of β-zearalenonol to zearalenone in the biosynthetic pathway [10]. As a putative isoamyl alcohol oxidase, the IAO protein may be involved in electron/proton transport [11].

Reverse transcription polymerase chain reaction was performed to demonstrate that the *iao* gene is expressed during patulin biosynthesis. Primers were designed to encompass two introns to confirm that RNA was amplified, rather than DNA, because the bands detected on the agarose gel would have had a larger size if the products amplified had been DNA (Fig. 3). As an additional check, bands were cut out of the gel, purified, and sequenced by symmetrical amplification in duplicate (data not shown), confirming the sequence identity of the bands.

This is the first report of an isoamyl alcohol oxidase gene in a species of the genus *Penicillium*. It is presently unknown what role it plays. Future research is under way to determine the importance of the *iao* gene in the patulin biosynthetic pathway by making and testing knockout mutants.

Acknowledgments Special thanks are given to Amy E. McGovern for excellent technical assistance. Names of equipment and chemical supplies are necessary to report factually on experimental methods; however, the USDA neither guarantees nor warrants the standard of the products, and the use of the name by USDA implies no approval of the products to the exclusion of others that may also be suitable.

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